



# Comparative analysis of the immune responses induced by native versus recombinant versions of the ASP-based vaccine against the bovine intestinal parasite *Cooperia oncophora*



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## ABSTRACT

The protective capacities of a native double-domain activation-associated secreted protein (ndd-ASP)-based vaccine against the cattle intestinal nematode *Cooperia oncophora* has previously been demonstrated. However, protection analysis upon vaccination with a recombinantly produced antigen has never been performed. Therefore, the aim of the current study was to test the protective potential of a *Pichia*-produced double-domain ASP (pdd-ASP)-based vaccine against *C. oncophora*. Additionally, we aimed to compare the cellular and humoral mechanisms underlying the vaccine-induced responses by the native (ndd-ASP) and recombinant vaccines. Immunisation of cattle with the native *C. oncophora* vaccine conferred significant levels of protection after an experimental challenge infection, whereas the recombinant vaccine did not. Moreover, vaccination with ndd-ASP resulted in a higher proliferation of CD4-T cells both systemically and in the small intestinal mucosa when compared with animals vaccinated with the recombinant antigen. In terms of humoral response, although both native and recombinant vaccines induced similar levels of antibodies, animals vaccinated with the native vaccine were able to raise antibodies with greater specificity towards ndd-ASP in comparison with antibodies raised by vaccination with the recombinant vaccine, suggesting a differential immune recognition towards the ndd-ASP and pdd-ASP. Finally, the observation that animals displaying antibodies with higher percentages of recognition towards ndd-ASP also exhibited the lowest egg counts suggests a potential relationship between antibody specificity and protection.

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## 1. Introduction

The economic impact of gastrointestinal nematode infections in ruminants has been extensively demonstrated over the years (Charlier et al., 2014). To date, control of these infections in livestock relies almost exclusively on the use of anthelmintic drugs, but the increasing spread of anthelmintic resistance worldwide illustrates the need for alternative control strategies (De Graef et al., 2013; Geurden et al., 2015). Successful vaccination against gastrointestinal nematodes with native and recombinant proteins in cattle and sheep (Meyvis et al., 2007; Besier et al., 2012; Nisbet et al., 2013; Vlamincx et al., 2015; Gonzalez-Hernandez et al., 2016), demonstrates that protein-based vaccination is a

promising alternative to the current control methods (Matthews et al., 2016). Recently, our group has shown that vaccination with native double-domain activation-associated secreted protein (ndd-ASP) obtained from the excretory-secretory material of the adult stages of the bovine intestinal nematode *Cooperia oncophora* (Borloo et al., 2013) provides protection both under experimental and natural conditions, as indicated by a decrease in the cumulative egg output of 91% and 59%, respectively (Vlamincx et al., 2015). Under natural conditions, vaccination of cattle with the ndd-ASP also resulted in a 65% reduction in pasture larval counts and a decrease in 82% of worm counts when compared with the control group (Vlamincx et al., 2015). Additional studies on the immune response elicited by vaccination with ndd-ASP have shown an increased antigen-specific antibody production, which correlated negatively with the egg output. Moreover, significant T-cell proliferation was induced in ndd-ASP vaccinated animals after in vitro stimulation with the antigen, which was positively

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correlated with a higher proportion of immature larval stages (Van Meulder et al., 2015). Despite these promising results achieved with the native antigen, recombinant production of the antigen would be an absolute requirement for the economic viability of the vaccine (Geldhof et al., 2007; Matthews et al., 2016). Therefore, the objectives of the present study were: (i) to produce and evaluate the protective capacity of a *Pichia*-produced version of the dd-ASP, and (ii) to compare the cellular and humoral responses induced by both native and recombinant versions of the antigen.

## 2. Materials and methods

### 2.1. Native and recombinant antigen production

Preparation of the native *C. oncophora* dd-ASP (ndd-ASP) was carried out as previously described (Borloo et al., 2013a). In short, excretory-secretory material of adult worms was collected and fractionated over a Superdex 200 16/70 size-exclusion chromatography column. The purity of the ndd-ASP fraction, which eluted first from the column, was checked by reducing and non-reducing one-dimensional gel electrophoresis (SDS-PAGE) and visualized by Simply-Blue SafeStain (Invitrogen, USA) staining. Afterwards, the protein band was excised and protein identity was further confirmed by mass spectrometry.

Recombinant double-domain ASP (pdd-ASP) was expressed in *Pichia pastoris* as follows: its coding sequence (Borloo et al., 2013b) was PCR-amplified and subsequently cloned into the pGEMt-Easy subcloning vector (Promega, USA) according to the manufacturer's instructions. Following transformation into *Escherichia coli* DH5 $\alpha$  competent cells (Invitrogen), clone selection on X-gal plates and sequence verification, the pGEMt-Easy-nDD-ASP construct was linearized by *EcoRI*-*XbaI* digestion and inserted in the *Pichia* expression vector pPICZ $\alpha$  B (Thermo Fischer Scientific, USA). The resulting expression plasmid was used to transform *P. pastoris* strain KM071H (Invitrogen) by electroporation. Afterwards, individual clones growing on minimal plates were isolated and tested for secretion of pdd-ASP by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblotting with bovine anti-nDD-ASP antibodies. A clone expressing pdd-ASP was freshly grown on minimal plates and then used to inoculate a shake flask culture with buffer minimal glycerol complex medium (BMGY). After 48 h of growth at 29 °C, the cells were pelleted by centrifugation for 15 min at 1,000g followed by resuspension of the cells in buffered methanol complex medium (BMMY) and further growth at 29 °C. Every 24 h, extra methanol (0.5%) was added to the culture and after 96 h of induction the cells were finally pelleted by centrifugation for 15 min at 2,000g. The cell medium was harvested and filtered over a 0.22  $\mu$ m membrane, after which the supernatant was concentrated on a Centrprep YM10 (Millipore, USA), dialyzed to 50 mM sodium acetate pH 5.0 and desalted on a HiPrep 26/10 Desalting column (GE Healthcare Bio-Sciences, USA). This fraction was then applied to a Resource-S cation exchange column (GE Healthcare Bio-Sciences AB) equilibrated in 50 mM sodium acetate buffer (pH 5.0) and the bound pdd-ASP was eluted by employing a gradient from 0 up to 1 M sodium chloride (NaCl) in the same buffer. Fractions containing pdd-ASP were pooled and buffer-exchanged to PBS by gel filtration on a Sephadex G25-column (GE Healthcare Bio-Sciences). The protein profile of the obtained material was checked on a 12% reducing SDS-PAGE. Additionally, ndd- and pdd-ASP proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane for 1 h at 1 mA/cm<sup>2</sup> of gel surface followed by a 1 h blocking step in PBS +0.5% Tween80 (PBST). The membrane was afterwards incubated with ndd-ASP-specific antibodies (dilution 1/500) for 1 h, followed by extensive washing with PBST and incubation for 1 h with horseradish

peroxidase (HRP)-conjugated rabbit anti-bovine IgG1 antibody. 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, USA) served as a substrate. All steps were performed at room temperature.

### 2.2. Immunization experiments in cattle

All animal experiments were conducted in accordance with the European Union Animal Welfare Directives and VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guidelines for Good Clinical Practice, and ethical approvals to conduct the studies were obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2014/70, EC2015/40). Two vaccination studies were carried out in cattle as previously described (Van Meulder et al., 2015; Vlamincx et al., 2015).

The aim of study 1 was to investigate the protective capacity of the recombinant antigen. Twenty-one helminth-naïve male cross-breed Holstein calves (6–8 months of age) were randomly divided over three groups of seven animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). Subsequently, a second study was performed to analyse and compare the cellular responses induced by the native and recombinant antigens, where 12 helminth-naïve female crossbred Holstein calves (6–8 months of age) were randomly divided over three groups of four animals (QuilA control, ndd-ASP+QuilA and pdd-ASP+QuilA). For both studies, all animals were immunized three times i.m. in the neck at 3 week intervals. Control animals received 750  $\mu$ g of QuilA (Superfos Biosector, USA), while the animals in the ndd-ASP+QuilA and pdd-ASP+QuilA groups received 30  $\mu$ g of antigen in combination with 750  $\mu$ g of QuilA per immunization. All animals were challenged with a trickle infection of 25,000 L3s (1000 L3s/day; 5 days/week for 5 weeks), which started on the day of the third immunization, and were then euthanized 3 weeks after the last infection. Parasitological parameters (i.e. faecal egg and worm counts) were monitored as previously described (Van Meulder et al., 2015; Vlamincx et al., 2015).

### 2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

In Study 2, blood samples were collected weekly from the jugular vein using vacutainer tubes. PBMCs were isolated by Lymphoprep (Nycomed Pharma, Norway) gradient centrifugation. Additionally, mesenteric lymph nodes (LNs) of the small intestine were isolated at the time of necropsy, and LN mononuclear cells (MNCs) were isolated by homogenization through mechanical disruption of the tissue followed by Lymphoprep gradient centrifugation. After centrifugation, all mononuclear cell fractions were isolated, washed and counted prior to cell culture and flow cytometric analysis.

### 2.4. Flow cytometry

Cells were labelled in Flow Cytometry Staining Buffer (eBioscience, USA) and all antibodies were used at the concentration recommended by the supplier. After incubation for 20 min with primary antibodies, the cells were washed twice prior to staining with fluorescently-labelled secondary antibodies. The cells were then incubated for an additional 20 min, washed and resuspended in PBS to be immediately analysed using a FACS Aria III flow cytometer (BD Biosciences, USA). Non-viable cells were excluded from the analysis based on their propidium iodide (Life Technologies, USA) uptake.

Primary antibodies used were: non-labeled CD3 (MM1A, IgG1), TCR $\gamma\delta$  (GB21A, IgG2b), CD21 (BAQ15A, IgM), CD8 (BAQ111A, IgM) (all from Monoclonal Antibody Center, Washington University, USA), CD4 (CC8, IgG2a) and CD335-IgG2b (AKS6, IgG1, kindly

provided by Prof. Dr. Anne K. Storset, Faculty of Veterinary Medicine of Oslo, Norway). Secondary antibodies used were: goat anti-mouse IgG1-V450 (BD Biosciences, USA), goat anti-mouse IgG2a-APC (Invitrogen), rat anti-mouse IgG2b-FITC (Southern Biotech, USA) and rat anti-mouse IgM-APC-Cy7 (Biolegend, USA).

### 2.5. Proliferation assays

The PBMCs and MNCs collected from blood and mesenteric LNs, respectively, in study 2 were used in proliferation assays using either  $^3\text{H}$ -thymidine ( $^3\text{HT}$ ) incorporation or PKH26 (Sigma–Aldrich, USA) fluorescence intensity reduction as a read-out as previously described (Gonzalez-Hernandez et al., 2016). For the  $^3\text{HT}$  uptake experiments, cells were seeded at  $1.25 \times 10^6$  cells/ml in a 96-well round-bottom plate (Thermo Scientific, USA) in complete medium (RPMI 1640 + GlutaMAX (Invitrogen, USA), 50 µg/ml of Gentamycin (Invitrogen, USA), 50 µM  $\beta$ -mercaptoethanol (Sigma–Aldrich, USA) and 10% FCS (Moregate, Australia)). Each well was either stimulated with medium alone, 5 µg/ml of ndd-ASP, 5 µg/ml of pdd-ASP or 1 µg/ml of ConA (Sigma–Aldrich, USA). Five days later, cells were pulsed with 1 µCi  $^3\text{HT}$  (Perkin Elmer, USA). After 18 h, cells were harvested and analysed with a 1450 Microbeta  $\beta$ -scintillation counter (Perkin Elmer).

For the Paul Karl Horan (PKH)26 experiments, PBMCs and mesenteric LN MNCs were collected 1 week after the last vaccination and at the time of necropsy, respectively, and labelled with PKH26 (Sigma–Aldrich, USA) as previously described (Gonzalez-Hernandez et al., 2016). After labelling, cells were seeded at  $2.5 \times 10^6$  cells/ml in complete medium in 96-well round-bottom plates, and either stimulated with medium alone, 5 µg/ml of ndd-ASP or 5 µg/ml of pdd-ASP. After 5 days of culture, the cells were harvested, stained with monoclonal antibodies and analysed by flow cytometry. ModFit LT software (Verity Software House) was used to calculate the proliferation index (PI) for the different cell populations.

In order to measure cytokine production during the proliferation of bovine PBMCs and mesenteric LN MNCs, supernatants of the abovementioned cultures were collected at time of harvesting after 5 days of culture. ELISA for detection of IFN $\gamma$  and IL-4 was performed by coating capture monoclonal antibodies in carbonate buffer (pH 9.6) on 96-well Maxisorp (Nunc, USA) plates at a concentration of 2 µg/ml and 4 µg/ml, respectively. Plates were incubated with blocking buffer (2% BSA in PBS supplemented with 0.05% Tween20 (Sigma–Aldrich)) for 1 h. Afterwards, 25 µl or 50 µl of supernatant (for detection of IFN $\gamma$  and IL-4, respectively) were placed in each well and blocking buffer was used to adjust the volume to 100 µl per well. Biotinylated monoclonal antibodies raised against IFN $\gamma$  or IL-4 were added at 2 µg/ml. Streptavidin–HRP (Sigma–Aldrich, USA) was used as a conjugate and 3,3',5,5'-tetramethylbenzidine (TMB) (Thermo Scientific, USA) served as substrate. OD was measured at 450 nm. All antibodies were kindly provided by Prof. Jayne Hope (The Roslin Institute, UK).

### 2.6. Antibody responses

For both studies, sera were collected before the first immunisation and 1 week after each immunisation. In addition, a 1 cm $^2$  piece of the small intestine (complete intestinal wall) at 3 m from the pylorus was collected at the time of necropsy. Small intestinal extracts were obtained by incubating ground frozen tissue in PBS for 2 h at 4° while shaking, and the protein concentration was determined with a bicinchoninic acid (BCA) assay kit (Pierce, USA). Samples were subsequently centrifuged for 15 min at 16000g and the supernatants collected.

The systemic and intestinal IgG1, IgG2 and IgA levels against the *C. onchophora* ndd-ASP were then determined by ELISA. ndd-ASP was coated on 96-well Maxisorp plates (Nunc, USA) at a concentration of 0.5 µg/ml in carbonate buffer (pH 9.6). After 1 h incubation with blocking buffer (2% BSA-PBS-0.05%Tween20), either 100 µg of intestinal extract or 100 µl of serum, 1/200-diluted in blocking buffer, were added in duplicate and incubated for an additional hour. After washing, sheep anti-bovine IgG1, IgG2 and IgA (Bio-Rad, USA) coupled to horseradish peroxidase (HRP) were used as conjugates (dilution 1/500). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Roche, Switzerland) served as a substrate. The OD was measured at 405 nm with a reference wavelength at 492 nm.

In addition, inhibition ELISA (iELISA) assays were performed to evaluate and compare the specificity of the antibodies raised against the native and recombinant versions of the dd-ASP. For both studies, pooled small intestinal extracts from the ndd-ASP +QuilA vaccinated animals were incubated at a final concentration of 200 µg/ml for 1 h at room temperature with different concentrations of either ndd-ASP or pdd-ASP, ranging from 0 to 1000 pmol/ml. The pre-incubated samples were afterwards tested in duplicate in a ndd-ASP coated plate as previously described for the conventional ELISA. Finally, an additional iELISA for the IgG1 isotype was performed for study 2 where intestinal extract from ndd-ASP +QuilA vaccinated animals was pre-incubated with pdd-ASP, and tested afterwards on a ndd-ASP coated plate as described for the conventional ELISA.

### 2.7. Statistical analysis

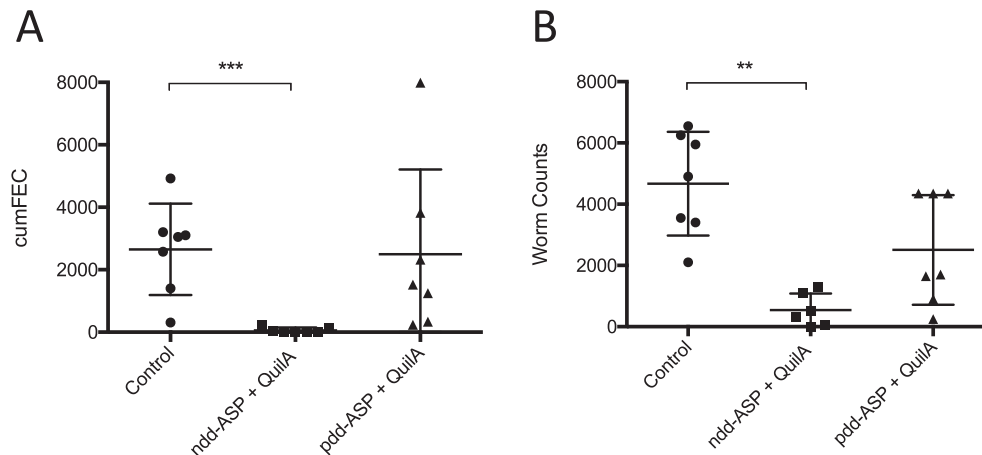
Statistical analyses were carried out using GraphPad Prism software. A non-parametric Kruskal–Wallis test followed by a Dunn's multiple comparison test were used to determine significant differences in parasitological parameters, antibody responses and  $^3\text{HT}$  assays. For the latter, an additional Analysis of Covariance (ANCOVA) (SPSS, IBM SPSS statistics version 23.0) was used to evaluate the impact of the outcome of one specific week in the results of the consecutive week. To determine significant differences in cytokine production and proliferation between the various cell subsets in the PBMCs and MNCs, a non-parametric Mann–Whitney test was used.  $P \leq 0.05$  was considered significant.

## 3. Results

### 3.1. Evaluation of a *C. onchophora* recombinant-ASP protein as a potential vaccine candidate

Following expression and purification of the pdd-ASP, the correct size and purity of the protein was verified by SDS–PAGE analysis (Supplementary Fig. S1), showing uniform glycosylation in contrast to its native counterpart which displays more heterogeneity in its N-glycan profile. Afterwards, the protein band was excised and the protein identity was further confirmed by mass spectrometry (data not shown).

This material was subsequently used in a first study to evaluate the protective capacities of the recombinantly produced *C. onchophora* dd-ASP protein in comparison with the native antigen. As indicated in Fig. 1, animals vaccinated with the ndd-ASP+QuilA vaccine showed a reduction of 99% in cumulative faecal egg counts (cumFEC) and 88% decrease in worm counts compared with the control vaccinated group, confirming previous findings (Vlaminck et al., 2015). Lower cumFEC and worm counts were observed following vaccination with pdd-ASP+QuilA, although differences did not reach statistical significance (Fig. 1A and B).



**Fig. 1.** Effect of vaccination with native (n) versus recombinant (p) double-domain activation-associated secreted protein (dd-ASP) of *Cooperia oncophora* on egg and worm counts. For animals vaccinated with QuilA alone (Control), ndd-ASP+QuilA and pdd-ASP+QuilA, (A) cumulative number of eggs per gram of faeces (cumFEC) and (B) worm counts in Study 1 were determined three times each week during the trickle infection and at the time of necropsy, respectively. Graphs show the mean cumFEC or worm counts in each group  $\pm$  S.E.M. Statistically significant differences compared with control vaccinated animals are indicated with \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.005$ ), \*\*\* ( $P \leq 0.001$ ).

### 3.2. Antibody responses after vaccination with native and recombinant versions of the vaccine

Vaccination with both ndd-ASP+QuilA and pdd-ASP+QuilA vaccines resulted in a significant increase in ndd-ASP-specific IgG1 and IgG2 levels in sera compared with QuilA control animals, while a significant increase in systemic IgA levels could only be detected after vaccination with ndd-ASP+QuilA (Fig. 2A). In the small intestinal mucosa, vaccination with the ndd-ASP+QuilA vaccine also resulted in a significant increase in ndd-ASP-specific IgA, IgG1 and IgG2 levels, whereas vaccination with pdd-ASP+QuilA only induced a significant increase in the ndd-ASP-specific IgG2 level, and higher but not significant levels of IgG1 or IgA (Fig. 2B).

To determine possible differences in the specificity of antibodies induced by vaccination with ndd-ASP+QuilA, pooled small intestinal extracts collected at necropsy were subjected to an iELISA. Pre-incubation of the small intestinal extracts with pdd-ASP failed to inhibit the binding of the IgG1, IgG2 and IgA antibodies induced in ndd-ASP+QuilA vaccinated animals, while pre-incubation with ndd-ASP almost completely inhibited IgG1, IgG2 and IgA binding to ndd-ASP (Fig. 2C).

### 3.3. Analysis and comparison of the cellular vaccine-induced responses by the native and recombinant vaccines

The aim of the second study was to confirm the results obtained in study 1 in terms of antibody responses, and to compare the differences in the vaccine-induced cellular responses between both native and recombinant vaccines. To do so, an experimental design similar to study 1 was carried out where animals were challenged post-vaccination and cellular, humoral and parasitological parameters were monitored.

To monitor cellular responses during the vaccination period, PBMCs were isolated on a weekly basis to measure antigen-specific proliferation in vitro. Results are shown in Fig. 3A as a stimulation index (SI) (= counts per minute (CPM) antigen stimulated cells/CPM medium stimulated cells). After in vitro restimulation of PBMCs with the vaccine antigens (ndd-ASP for the ndd-ASP+QuilA and Control groups, and pdd-ASP for the pdd-ASP+QuilA) antigen-specific proliferation was found in the ndd-ASP+QuilA group (Fig. 3A). Proliferation in the ndd-ASP+QuilA group was the highest and significantly different from control animals at week 4 after the first vaccination. From week 5 onwards, cellular proliferation continued to be significantly higher in the ndd-ASP+QuilA group compared with control and pdd-ASP-vaccinated animals.

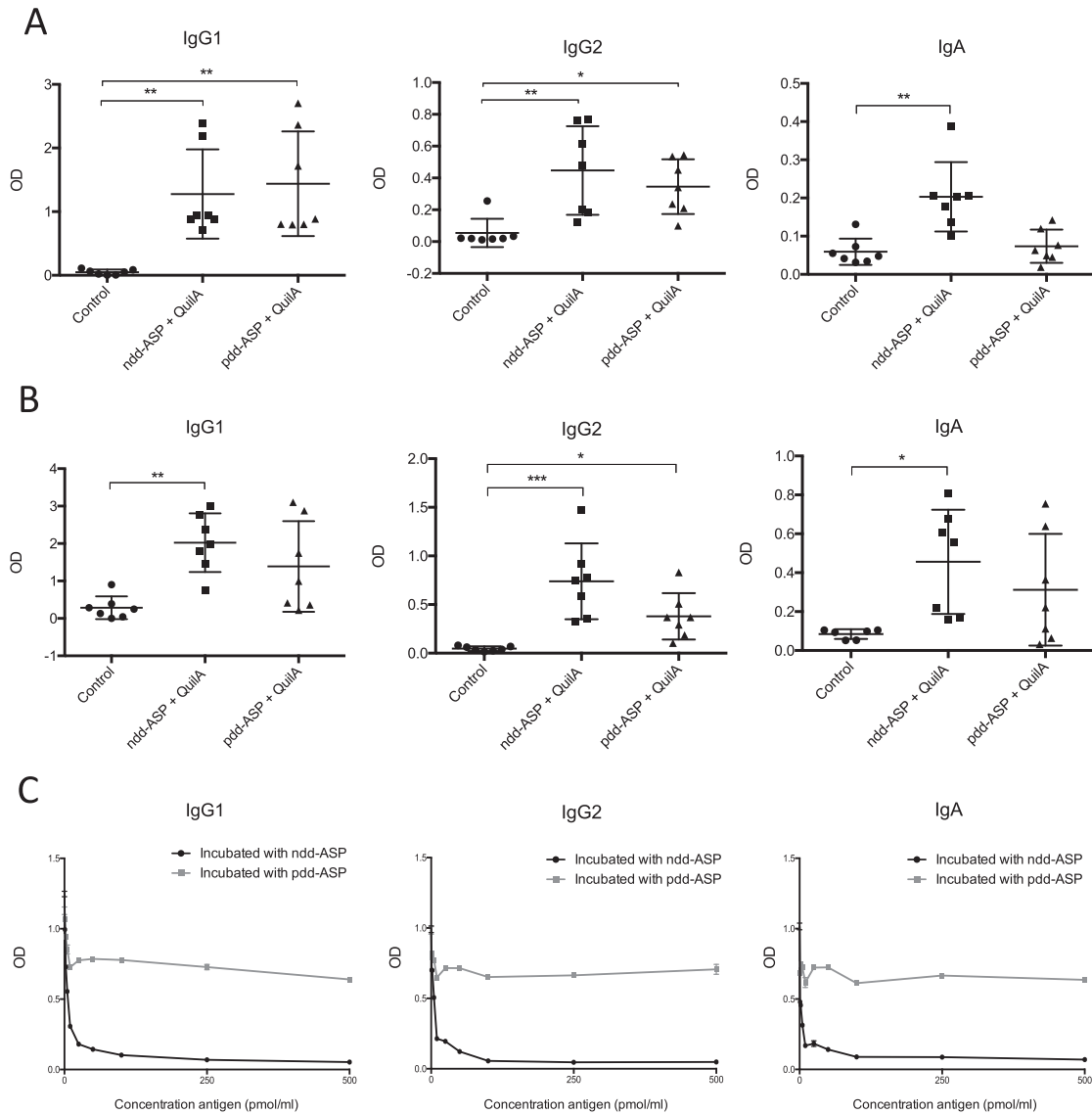
One week after the third vaccination (week 7), vaccine antigen-specific proliferation could also be observed in animals vaccinated with pdd-ASP+QuilA (Fig. 3A). Additionally, an ANCOVA test was performed to assess the potential effect of the results of one week in the following week with no significant results.

To determine the phenotype of the antigen-specific PBMCs that proliferated during the vaccination period after in vitro stimulation, PBMCs were isolated at week 7, labelled with PKH and cultured either in the presence of medium alone or 5  $\mu$ g/ml of the vaccine antigen. After 5 days, cells from all groups were harvested and analysed by flow cytometry. Although the thymidine data showed antigen-specific proliferation at week 7 in the pdd-ASP+QuilA group, no proliferating cell population could be identified in the PKH assay (Fig. 3B and C). Whether this is due to a population of cells for which no specific markers were included in the PKH assay is currently unclear. On the other hand, significant antigen-specific proliferation of CD4-T cells after in vitro stimulation with ndd-ASP was observed in animals vaccinated with ndd-ASP+QuilA when compared with the medium-stimulated cells (Fig. 3D).

To characterize the phenotype of the cellular response in the small intestine following vaccination and challenge infection, LNs from all animals were collected at time of necropsy and MNCs were stimulated in vitro as previously described for PBMCs. For all groups, proliferation of  $\gamma\delta$ -T cells, CD4-Tcells, natural killer (NK) cells and non-T, non-B non-NK cells (CD3<sup>+</sup>/CD21<sup>+</sup>/CD335<sup>+</sup>) was significantly higher after re-stimulation with ndd-ASP (Fig. 4A–C), suggesting that the challenge infection had an effect on the local immune response. However, proliferation after re-stimulation with ndd-ASP was significantly higher in animals vaccinated with ndd-ASP+QuilA for CD4-T cells and CD8-T cells when compared with the control and pdd-ASP+QuilA groups (Fig. 4D). ELISAs performed to detect the presence of either IL-4 or IFN $\gamma$  in the culture supernatants showed that in vitro restimulation with ndd-ASP induced a significantly higher production of both IL-4 and IFN $\gamma$  in all groups when compared with the medium stimulated cells (Fig. 4E and F).

Identical to study 1, the systemic and mucosal antibody responses were measured both in a quantitative and qualitative way. The levels of IgG1 and IgG2, but not of IgA, ndd-ASP-specific antibodies in serum were significantly higher in animals



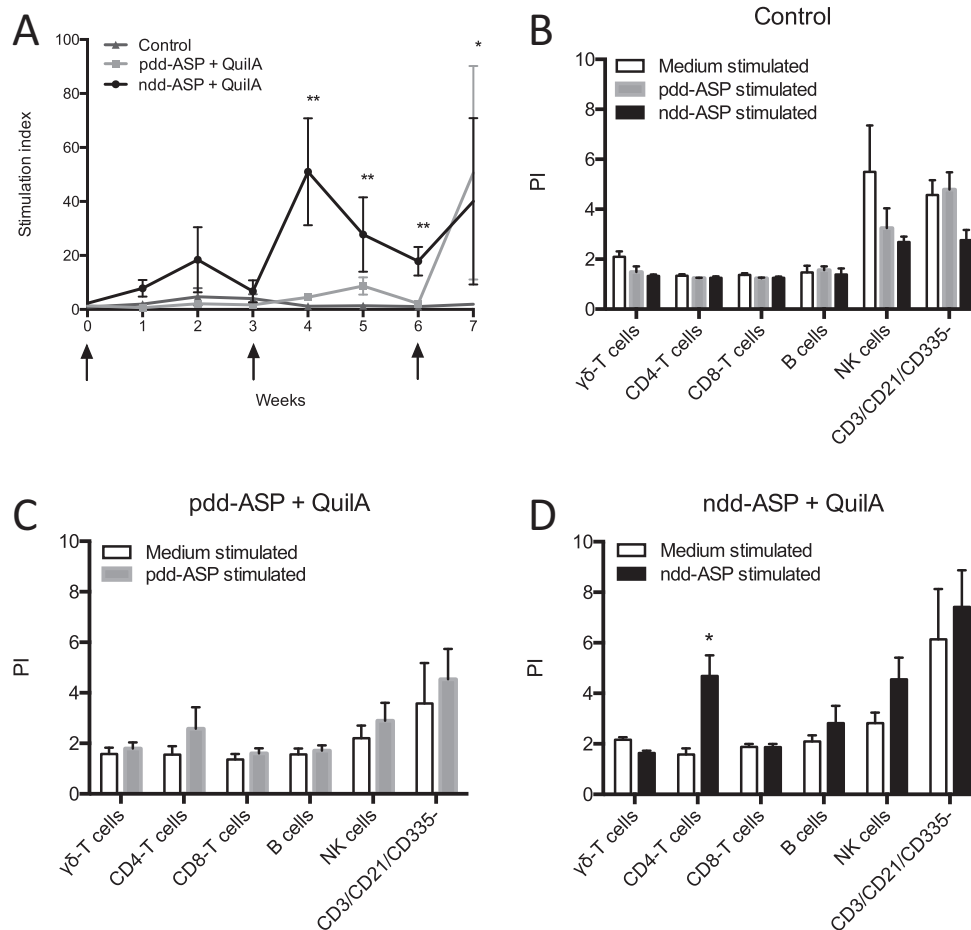


**Fig. 2.** Antibody responses in serum and mucosa after vaccination with the native and recombinant vaccines. In cattle study 1, (A) serum from 1 week after the second vaccination and (B) mucosal samples were collected from all animals and used for the detection of *Cooperia oncophora* native double-domain activation-associated secreted protein (ndd-ASP)-specific IgG1, IgG2 and IgA antibodies by ELISA. The graphs show the individual absorbance values within each group and the mean  $\pm$  S.E.M. Statistically significant differences are indicated with \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.005$ ), \*\*\* ( $P \leq 0.001$ ). (C) Small intestinal extracts from calves vaccinated with ndd-ASP+QuilA were pre-incubated with either ndd-ASP or recombinant (p)dd-ASP and evaluated in an inhibition ELISA for IgG1, IgG2 and IgA isotypes. Graphs were generated from pooled samples analysed in duplicate.

vaccinated with both ndd-ASP+QuilA and pdd-ASP+QuilA (Supplementary Fig. S2A). In the mucosa, IgG1 levels were significantly higher in ndd-ASP+QuilA vaccinated animals but not in pdd-ASP+QuilA vaccinated animals (Supplementary Fig. S2B). In contrast, animals vaccinated with pdd-ASP+QuilA showed a significant increase in antigen-specific IgG2 and IgA levels. This increase, although not significant, could also be observed in animals vaccinated with ndd-ASP+QuilA (Supplementary Fig. S2B). Finally, iELISAs for IgG1, IgG2 and IgA were performed with intestinal extracts from animals vaccinated with ndd-ASP+QuilA with identical results to those observed in Study 1 (Supplementary Fig. S2C).

Finally, in terms of parasitological parameters, no reduction could be observed for animals vaccinated with pdd-ASP+QuilA (Supplementary Fig. S3). On the other hand, three out of four animals in the ndd-ASP+QuilA group did show a clear reduction in cumEPG (Supplementary Fig. S3A) but, importantly, one animal

was not protected. In order to investigate whether there was a correlation between antibody specificity and protection, an IgG1 iELISA was performed on intestinal extract from the individual animals vaccinated with ndd-ASP+QuilA, in which the extracts were pre-incubated with pdd-ASP and subsequently tested for binding a ndd-ASP coated plate. Ideally, pdd-ASP would be able to display similar inhibitory capacities towards antibodies as the ndd-ASP, and pre-incubation of antibodies from ndd-ASP-vaccinated animals with pdd-ASP should prevent them from binding the ndd-ASP present on the plate. The results of this assay, as shown in Fig. 5A, indicated firstly that pdd-ASP is unable to inhibit antibodies from binding to the ndd-ASP. In addition, results show that there was a variation in the specificity of the antibodies induced in the different animals and that the antibodies present in the non-protected animal showed the lowest specificity toward ndd-ASP (Fig. 5B). Such a potential relationship was not observed for cellular proliferation or antibody levels (results not shown).



**Fig. 3.** Systemic cellular responses following vaccination with native (n) and recombinant (p) dd-ASP vaccines. (A) During the vaccination period, peripheral blood mononuclear cells (PBMCs) were isolated weekly, re-stimulated with either medium alone or the antigen used in the vaccine formulation, and the proliferation was determined based on H-thymidine ( $^3\text{H}$ ) incorporation. The graph shows the mean stimulation index  $\pm$  S.E.M. for all vaccinated groups ( $n = 4$ ), where the arrows indicate the time points at which the animals were immunised. (B–D) Seven weeks after the first vaccination, PBMCs from all groups were stimulated for 5 days with medium or the vaccine antigen, stained with Paul Karl Horan (PKH) to determine the proliferation with flow cytometry. The mean proliferation indices (PI)  $\pm$  S.E.M. of (B) QuilA Control ( $n = 4$ ), (C) pdd-ASP+QuilA ( $n = 4$ ), and (D) ndd-ASP+QuilA ( $n = 4$ ) vaccinated animals were calculated and compared with those of the control group. Statistically significant differences are indicated with \* ( $P \leq 0.05$ ), \*\* ( $P \leq 0.005$ ).

#### 4. Discussion

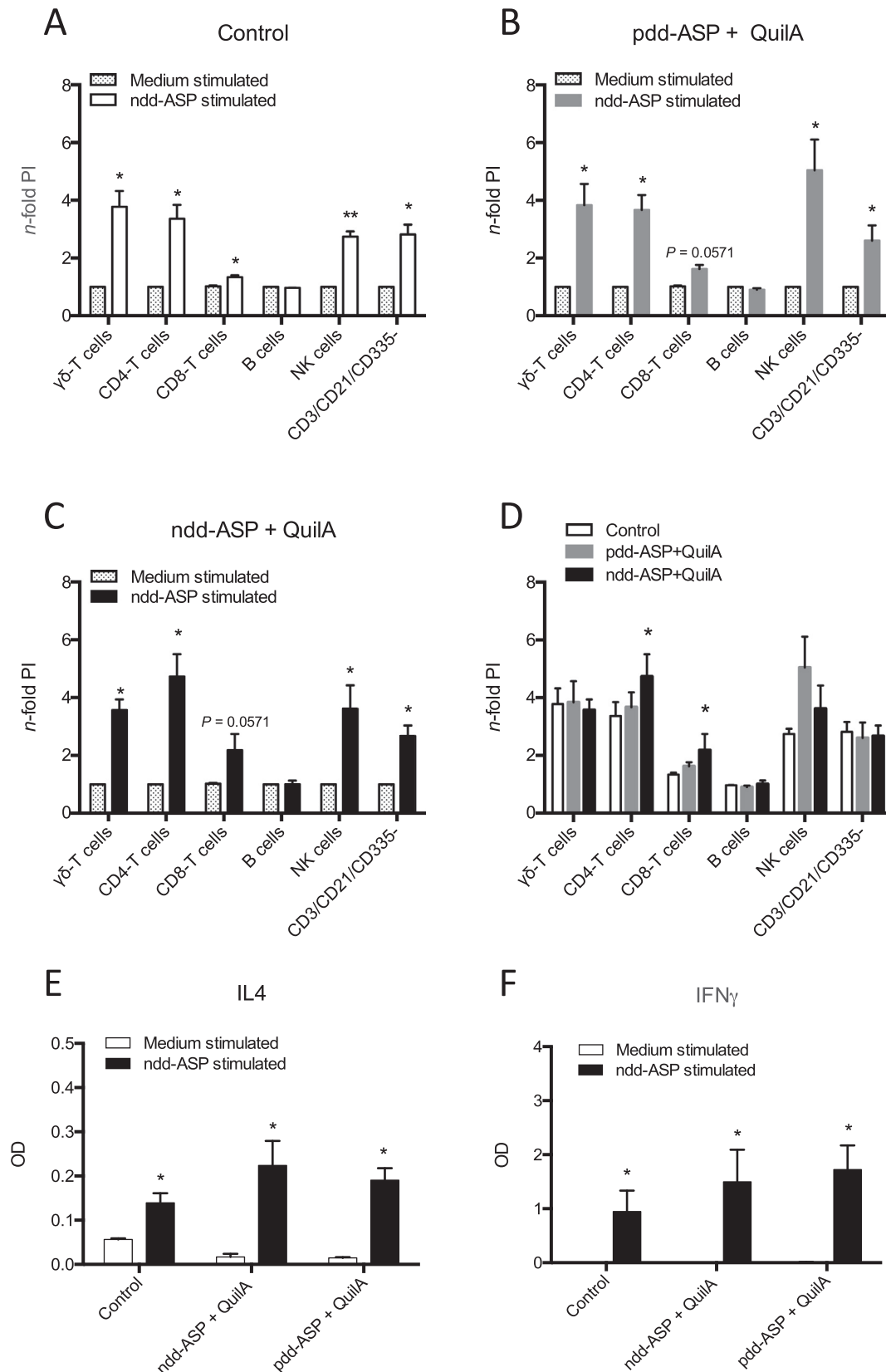
Similar to our previous findings (Vlaminck et al., 2015), vaccination with the native antigens (ndd-ASP) provided protection against an experimental infection with *C. oncophora*. Inducing a protective response with recombinant antigens has proven to be extremely challenging for most of the anthelmintic vaccines tested to date (Geldhof et al., 2007; Gonzalez-Hernandez et al., 2016). The present study shows that this is also the case for the dd-ASP vaccine against *C. oncophora*, as vaccination of calves with a *Pichia*-produced version of the antigen (pdd-ASP) did not confer any protection.

Previous studies on vaccine-induced immunity using the native antigen against *C. oncophora* revealed the induction of a strong antigen-specific cellular response in the small intestinal mucosa, mainly characterized by the presence of antigen-specific  $\alpha\beta$ - and  $\gamma\delta$ -T cells (Van Meulder et al., 2015). In line with this, the present study demonstrates that vaccination of calves with ndd-ASP+QuilA, but not with pdd-ASP+QuilA or QuilA alone, resulted in an increased systemic memory response from the second vaccination onwards, which was characterized by the induction of antigen-specific CD4-T cell proliferation. Although proliferation of almost all cell populations could be detected for all groups in the

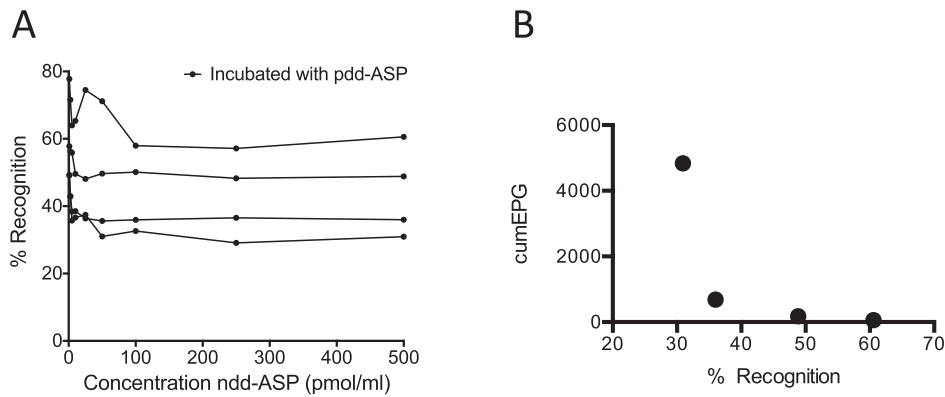
mesenteric LNs, the magnitude of this response, and more specifically of the CD4-T cell population, was higher in animals vaccinated with the native antigen. These results not only agree with the findings of Van Meulder et al. (2015), but also resemble the naturally acquired immunity to *C. oncophora* where an augmented CD4-T cell frequency has been observed both systemically and at the site of infection (Kanobana et al., 2003).

While immunity against helminth infections has been commonly associated with a Th2 response, the production of both IL-4 and IFN $\gamma$  by mesenteric lymphocytes in all groups after re-stimulation with ndd-ASP suggests a combined Th1/Th2 type response. The observation of a mixed Th1/Th2 response against a helminthic infection is not novel and has been extensively documented for other helminth species such as *Fasciola hepatica* (Moreau et al., 2010), *Trichinella spiralis* (Ilic et al., 2011) and *Ostertagia ostertagi* (Mihi et al., 2014).

In terms of humoral responses, the overall results indicated that both native and recombinant vaccines induce the production of antigen-specific IgG1, IgG2 and IgA antibodies. Nonetheless, inhibition ELISAs for all isotypes showed that the antibodies induced by the ndd-ASP preferentially bind to the ndd-ASP and not to the recombinantly produced version of the antigen (pdd-ASP), and vice versa (data not shown). Whether this difference in antibody



**Fig. 4.** Mucosal cellular responses following vaccination with native (n) and recombinant (p) dd-ASP vaccines. At the time of necropsy, mononuclear cells (MNCs) were isolated from the small intestinal lymph nodes and their proliferative capacity was evaluated after in vitro stimulation with ndd-ASP. Mean *n*-fold proliferation index (PI)  $\pm$  S.E.M. is shown individually for (A) Control (*n* = 4), (B) ndd-ASP+QuilA (*n* = 4) and (C) pdd-ASP+QuilA (*n* = 4) vaccinated animals. (D) Additionally, *n*-fold PI between all groups after ndd-ASP stimulation were compared and are shown as mean  $\pm$  S.E.M. Supernatants of the previous cultures were collected and the production of (E) IL-4 and (F) IFN $\gamma$  were determined. Both graphs show the mean OD of either IL-4 or IFN $\gamma$  from medium or antigen-stimulated supernatants for each group (*n* = 4)  $\pm$  S.E.M. Statistically significant differences are indicated with \* (*P*  $\leq$  0.05).



**Fig. 5.** Correlation between protection and antibody specificity. Small intestinal extracts from animals vaccinated with native (n)dd-ASP+QuilA in study 2 were individually tested in an inhibition ELISA (iELISA) by pre-incubation with recombinant (p)dd-ASP and later evaluation on an ndd-ASP coated plate. (A) The graph shows the individual percentage (%) of recognition for each animal. (B) Correlation between cumulative faecal egg count (cumFEC) values and antibody percentage of recognition in the animals vaccinated with ndd-ASP+QuilA.

specificity is important in conferring protection is still unclear. However, it is interesting to note that the only animal with high cumFEC from the ndd-ASP vaccinated group raised antibodies with the lowest specificity towards ndd-ASP, indicating the existence of a negative correlation between antibody specificity and parasitological parameters. This suggests an important role for antibodies in the protective immune response, an observation that has also been previously made for naturally acquired and vaccine-induced immunity against *C. oncophora* (Kanobana et al., 2001; Van Meulder et al., 2015).

Overall, the observation that both cellular and humoral responses differ between animals vaccinated with either the native or the recombinant form of the dd-ASP antigen are in line with our previous observations on vaccine-induced immunity against *O. ostertagi* (Gonzalez-Hernandez et al., 2016). This differential immune recognition is possibly caused by differences between native and recombinant proteins in terms of protein conformation and/or the associated glycan structures. ASPs belong to the cysteine-rich secretory/antigen 5/pathogenesis-related 1 (CAP) superfamily and have been suggested to play a role in immune evasion, although their exact biological functions remain unknown. As the name itself suggests, proteins belonging to this family are rich in cysteine residues that form several disulphide bridges, resulting in a highly conserved  $\alpha$ - $\beta$ - $\alpha$ -sandwich structure (Gibbs et al., 2008). The exact constitution of these disulphide bonds has only been obtained based on three-dimensional crystallographic data from recombinantly expressed material (Asojo, 2011; Borloo et al., 2013b), and whether these bridges are actually present in the native ASPs remains unclear. However, what seems evident is that protein folding is essential for mounting of a proper immune response, as demonstrated by the abolition of all vaccine-induced immune responses (both cellular and humoral) when vaccinating animals with an unfolded version of native *O. ostertagi* ASP1 protein (Gonzalez-Hernandez et al., 2016). In addition, structural analysis has revealed differences in the glycans present on the native and recombinant versions of the dd-ASP antigen (unpublished data). Potentially these differences influence the immunogenicity of the antigens. Therefore, further studies with non-glycosylated forms of the antigens are necessary to provide further insights on the importance of these glycans.

Taken together, the outcome of the present study shows that vaccination of calves with a protective ndd-ASP+QuilA vaccine, but not with its recombinant counterpart, results in a strong cellular memory response mainly characterized by the presence of antigen-specific CD4-T cells in both blood and intestinal mucosa. Although both ndd-ASP and pdd-ASP-based vaccines induced

similar levels of antibodies, the specificity of the antibodies raised by both antigens clearly differed. The possible structural differences that impede the recombinant vaccine to mount a protective immune response remain unknown and form the basis of our current research.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2017.07.002>.

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